Preclinical Toxicology and Anticholangiocarcinoma Activity of Oral Formulation of Standardized Extract of *Zingiber Officinale*

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ABSTRACT

Cholangiocarcinoma (CCA) remains a significant public health problem in Thailand. New effective and safe drugs are urgently needed. *Zingiber officinale* Roscoe (ZO) is a widely used medicinal plant for the treatment of several ailments, and the animal study suggests a potential anti-CCA activity. The present study aimed to develop the oral formulation of standardized extract of ZO and investigate toxicological profiles (acute, repeated dose, and chronic toxicity), including anti-CCA activity of the ZO formulation. The oral pharmaceutical formulation of the standardized ZO extract was successfully developed with an acceptable level of contamination and physicochemical and pharmaceutical properties. Acute, subacute, and chronic toxicity tests were conducted in healthy Sprague Dawley rats according to the OECD guidelines. The results showed no evidence of toxicity and death in the acute and subacute toxicity testing with the maximum tolerated dose (MTD) of 5000 and 2000 mg/kg body weight, respectively. Chronic toxicity revealed MTD and No-Observed-Adverse-Effect level (NOAEL) of 1000 mg/kg body weight. The anti-CCA activity was evaluated in CCA-xenografted mouse model. The formulated ZO powder was fed to animals daily for 30 days. Significant anti-CCA activity on tumor growth inhibition and prolongation of survival time were demonstrated at the high (2000 mg/kg body weight) and moderate (1000 mg/kg body weight) dose levels. Further investigation to elucidate molecular targets of action of ZO against CCA cells is encouraged.

Introduction

CCA or bile duct cancer is the second most common and predominant type of liver cancer [1]. The epidemiology of CCA varies in Asian countries but is commonly found in China, Korea, and Thailand. The highest incidence worldwide is reported from the northeastern region of Thailand (85 *per* 100 000 *per* year) [2]. Surgery is the only potentially curative treatment for CCA. However, 5-year survival rates after acquired surgery of patients with distal extrahepatic, intrahepatic, and hilar CCA are relatively low, (*i.e.,* 27–37%, 22–44%, and 11–1%, respectively) [3]. Standard chemotherapeutic drugs for CCA include 5-fluorouracil, cisplatin, gemcitabine, and doxorubicin [4, 5], but their clinical efficacy when used as single drugs or combination therapy remains unsatisfactory [6–8]. Effective alternative chemotherapeutic drugs are urgently needed.

Several natural compounds have been investigated for their potential treatment of CCA. *Zingiber officinale* Roscoe (ZO), commonly known as ginger, belongs to the Zingiberaceae family, is a common condiment for various foods and beverages. It has been used in Ayurvedic and Chinese medicine. In China, the rhizome of

ABBREVIATIONS

CCA	Cholangiocarcinoma
DMN	Dimethylnitrosamine
MTD	Maximum tolerated dose
NOAEL	No-Observed-Adverse-Effect level
OECD	Organisation for Economic Cooperation and Development
OV	*Opisthorchis viverrini*
ZO	*Zingiber officinale* Roscoe

ZO is used for many purposes [9]. The U. S. Food and Drug Administration recommends ginger as a "generally recognized as safe" list, although it can interact with some medications such as warfarin. Diverse pharmacological activities of ZO have also been demonstrated including anti-inflammatory, antioxidant, antimicrobial, analgesic, gastro-protective, antirheumatic, and anticancer activities [10–18]. Results from the acute and subacute toxicity testing indicate satisfactory safety profiles of the ethanolic extract of ZO with no significant toxicity at the MTD of 5000 mg/kg body weight [19]. The extract exhibited promising anti-CCA activity in OV/DMN-induced CCA hamster and CCA-xenograft nude mouse models with a significant reduction in tumor volume and prolongation of survival time compared with the control group [19, 20]. The present study aimed to confirm the safety and anti-CCA activity of the oral pharmaceutical formulation of the standardized extract of ZO before further clinical investigation.

Results

The yield of the formulated standardized extract of the dried ZO rhizomes in 95% ethanolic was 5.5%. The optimal proportion of the formulated ZO and other ingredients was the mixture of standardized ZO extract powder, lactose (water-soluble filler), sodium lauryl sulfate (0.1% mg, as a wetting agent), and talcum (0.9% mg as a glidant) at the ratio of 33/66/1% (w/w/w). The percentage content of 6-gingerol in the formulated ZO was 2.4%. The chromatogram of separation is shown in ▶ **Fig. 1**.

All physicochemical and pharmaceutical properties, including the level of contamination of the formulated ZO powder, were acceptable according to the standard guidelines. The cumulative amount (mean ± SD) of 6-gingerol released from the formulated capsule is shown in ▶ **Fig. 2**. The ZO formulated capsule showed regular physical appearance, and the actual weight was maintained after accelerated condition for up to 6 mo.

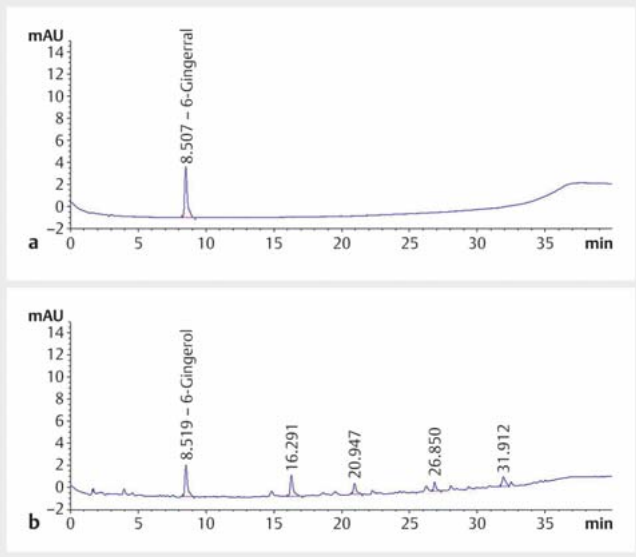
Acute oral toxicity: No evidence of toxicity and death was observed in all rats receiving the formulated ZO powder at the maximum dose of 5000 mg/kg, similarly to those receiving distilled water (untreated control). ▶ **Fig. 3a** shows the body weight change of rats during the observation period.

Subacute toxicity: The highest dose of orally administered formulated ZO powder that did not produce any sign of toxicity (changes in body weight, behavior, histopathology, and laboratory parameters) or mortality in all rats was 2000 mg/kg body weight. The control and ZO-treated groups had normal behavior with natural food and water consumption. The changes in body weights of rats in the treated and control groups were comparable (▶ **Fig. 3b**). The hematological and biochemical parameters generally lay within acceptable ranges, and there were no significant differences in the values between the control and treated male and female rats receiving all dose levels of ZO. Significant differences were observed only in some hematological (number of red cells, platelets, white cells, neutrophils, and lymphocytes) and biochemistry (cholesterol and triglyceride) parameters in treated rats at 500, 1000, and 2000 mg/kg body weight (▶ **Table 1**).

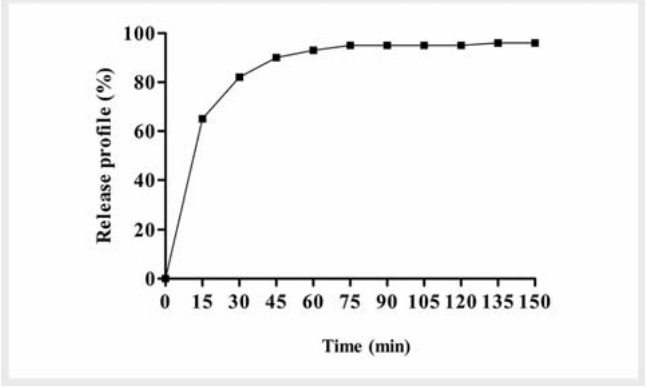
Chronic toxicity: The MTD level of formulated ZO powder following the 12-month repeated doses was 1000 mg/kg body weight. No treatment-related serious adverse clinical appearances were found during the 12 mo observation period. There was no significant difference between the body weight, food, and water consumption of male and female rats at all dose levels (250, 500, and 1000 mg/kg body weight). The NOAEL was, therefore, 1000 mg/kg body weight. ▶ **Fig. 3c** shows the body weight change of rats during the observation period. The hematological and biochemical parameters generally lay within acceptable ranges, and in most cases, the values were comparable between the control and treated male and female rats receiving all dose levels of ZO. Significant differences were observed only in some hematological (hemoglobin, number of white blood cells, neutrophils, and lymphocytes) and biochemistry (cholesterol, triglyceride, and glucose) parameters in the treated rats at high (1000 mg/kg body weight) and medium (500 mg/kg body weight) dose levels.

Histopathological examination revealed no apparent effects of the formulated ZO power compared with untreated control (▶ **Table 2**). The only minimal degree of changes (+) in pulmonary macrophage accumulation, spotty necrosis, and chronic renal interstitial inflammation were observed in rats treated with high (1000 mg/kg body weight) and medium (500 mg/kg body weight) dose levels of the formulated ZO powder. At the high dose level, the accumulation of macrophage in the lung was found in 25% and 15% of male and female rats, respectively. Furthermore, this dose level also resulted in spotty necrosis in liver and renal interstitial inflammation in 20% and 10% of male and female rats. For the reproductive system, minimal level toxicity of vaginitis and epithelial necrosis was observed in 1 female rat each (5%) at high and medium dose levels. Besides, a minimum level of testicular atrophy and the absence of mature sperm in the epididymis duct were observed in 1 male rat each after the high dose level. No abnormality was detected in other organs and tissues.

Anti-CCA activity: No significant body weight change was observed in all mice treated with the formulated ZO powder. The high and moderate dose levels of ZO extract (2000 and 1000 mg/kg body weight, respectively) and cisplatin-treated groups significantly inhibited tumor growth compared with the untreated control group (▶ **Fig. 4** and **5a**). Metastatic nodules were found in the lungs of both the untreated and ZO treated mice at all dose levels. The survival time of mice treated with cisplatin, high-, and medium-dose levels of the formulated ZO powder was significantly prolonged compared with untreated control (mean ± SD: 72 ± 5, 70 ± 5, 68 ± 4 *vs.* 45 ± 3 days, respectively) (▶ **Fig. 5b**). The survival time (mean ± SD) in the low dose group was 50 ± 3 days.



▶ **Fig. 1** HPLC chromatogram of (**a**) standard markers 6-gingerol and (**b**) formulated ZO powder.



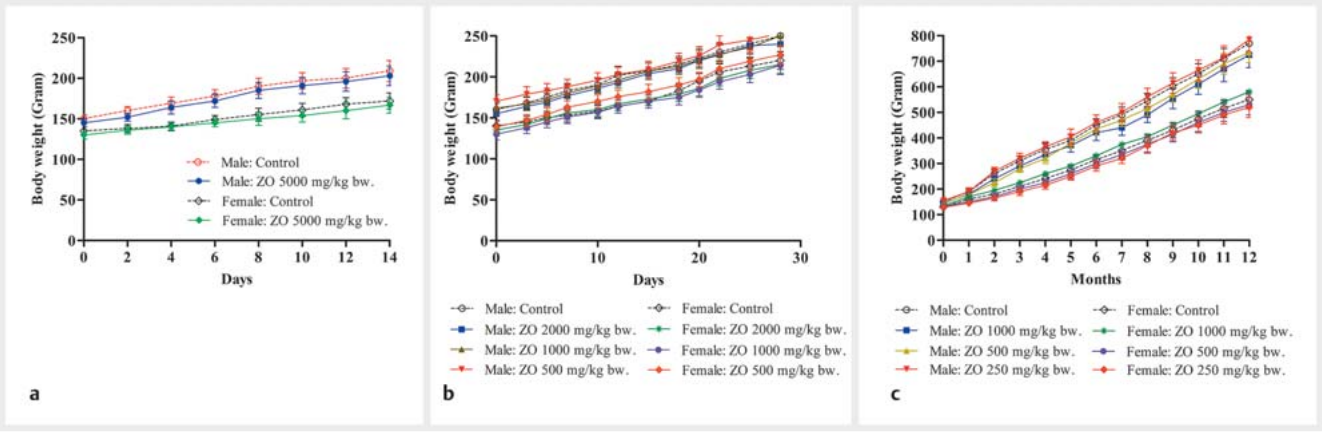
▶ **Fig. 2** Dissolution profile of 6-gingerol from the formulated ZO capsule.

Discussion

The current study successfully developed the oral pharmaceutical formulation of the standardized ZO extract with an acceptable level of contamination and physicochemical and pharmaceutical properties. The amount of the active component 6-gingerol was 2.4%. The optimal formulation mixture consisted of lactose, sodium lauryl sulfate, and talcum. Based on the results of stability testing, the stability of the formulated ZO capsule is expected to be maintained up to 2 y of production (2× the stability tested in the accelerated condition).

Toxicity testing generally confirmed the safety profile of the formulated powder at a single dose (acute toxicity), short-term repeated doses (subacute toxicity), and long-term repeated doses (chronic toxicity). Results indicated dose-dependent toxicity and mortality of the formulated ZO powder in rats. The MTD levels of the formulated ZO powder for acute, subacute, and chronic toxicity testing were 5000, 2000, and 1000 mg/kg body weight, respectively. The NOAEL was 1000 mg/kg body weight. There were no changes in general behavior, systematic anatomy, and organ weights. Interestingly, significant effects on hematological parameters (increase in hemoglobin, total white blood cells, neutrophils, and lymphocytes) and biochemistry parameters (decrease in cholesterol and triglyceride) were observed. This may suggest the potential use of ZO for hematological disorders as well as hypercholesterolemia. ZO has been used in traditional medicine in several countries for the treatment of arthritis and rheumatological conditions [21, 22], atherosclerosis, and hypercholesterolemia [23]. In preclinical studies, ZO was demonstrated to exhibit various pharmacological activities, including antihypercholesterolemic effects in rats [24], stimulation of hematopoiesis in zebrafish [25], and reducing lipid profile in rats [26]. In clinical studies, ZO was reported to lower triglyceride levels in hyperlipidemia patients, decrease total cholesterol and triglyceride, and increase HDL cholesterol in obese women, as well as increase WBC level in leukopenia induced-chemotherapy in cancer patients [27–29]. The increase in WBC numbers, especially lymphocytes, is associated with the induction of cellular immunity against infections and hematological disorders. Only a minimal level of histopathological changes was observed (▶ **Table 2**) in the lungs (macrophage accumulation), livers (spotty necrosis), kidneys (renal interstitial inflammation), testes (testicular atrophy), and vaginas and cervixes (epithelial necrosis) of rats receiving high- and medium-dose levels. The results of the chronic study demonstrated that the formulated ZO powder was well tolerated at 1000 mg/kg body weight without long-term or delayed toxicity. This data supported the observation of Rong et al. [30] regarding the safety profile of Japanese ginger at 2000 mg/kg body weight to male and female rats following the 35-day daily dose regimen.

Early reports suggested the potential role of ZO for the treatment of chronic myeloid leukemia [31], pancreatic cancer [32], ovarian cancer [33], and lung cancer [34]. In the present study, the anti-CCA activity of the formulated ZO powder was evaluated at 3-dose levels in CCA-bearing nude mice given daily for 30 days. High (2000 mg/kg body weight) and medium (1000 mg/kg body weight) dose levels significantly prolonged the survival time of mice by 55.6% and 51.1% of untreated control mice, respectively. Tumor growth was also significantly inhibited in both groups as well as the cisplatin-treated groups (▶ **Fig. 4** and **5 a**). In our previous investigation with the crude ethanolic extract of ZO (unformulated ZO), the anti-CCA activity was shown at all 3 investigated dose levels. The median survival time following high MTD (5000 mg/kg daily), moderate dose (3000 mg/kg daily), and low dose (1000 mg/kg daily) were prolonged by 70% (68 days), 57.5% (63 days), and 55% (62 days), respectively, compared with 40 days in untreated mice [20]. The potency of activity of the formulated ZO powder and unformulated ZO extract on survival time and tumor growth appears to be similar at equivalent dose levels. It is noted, however, that higher dose levels than 1000 mg/kg may be required for inhibitory activity of both the formulated and unformulated ZO powder on lung metastasis [20]. *In vitro* studies also demonstrated marked inhibitory activities of the ZO extract on cell invasion and angiogenesis of tube formation model in the CCA cell line [35]. In other reports, treatment with ZO or its bioactive components was shown to significantly reduce tumor metastasis. The ethanolic extract of ZO also



▶ **Fig. 3** Body weight changes in rats following an (**a**) acute, (**b**) subacute, and (**c**) chronic toxicity assessment and treatment with orally administered formulated ZO powder in rats.

▶ **Table 1** Significant differences in (a) hematological and (b) biochemistry parameters following subacute toxicity testing (repeat 28-day doses oral administration) in male and female rats following treatment with the formulated ZO (n = 5 rats per group). Data are presented as mean ± SD values.

Parameters	Sex	Groups			
		ZO formulation (mg/kg body weight)			Untreated control
		2000	1000	500	
(a) Hematological parameters					
RBC (10^6/μL)	Male	10.5 ± 0.9*	9.2 ± 0.6	9.0 ± 0.8	8.9 ± 0.6
	Female	10.1 ± 1.1*	8.3 ± 0.5	8.1 ± 0.6	7.9 ± 0.5
PLT (10^3/μL)	Male	1.015 ± 154*	996 ± 113*	867 ± 99	829 ± 79
	Female	991 ± 88*	975 ± 76*	846 ± 71	886 ± 94
WBC (10^3/μL)	Male	17.2 ± 2.9*	17.1 ± 2.0*	16.6 ± 1.3*	13.3 ± 1.5
	Female	15.5 ± 1.8*	14.9 ± 1.8*	15.3 ± 2.2*	10.2 ± 1.1
N (10^3/μL, %)	Male	3.9 ± 0.5*	3.8 ± 0.4*	3.8 ± 0.7*	2.7 ± 0.6
	Female	3.1 ± 0.5*	2.8 ± 0.3*	2.8 ± 0.5*	1.9 ± 0.4
L (10^3/μL, %)	Male	13.5 ± 1.2*	13.2 ± 0.9*	12.7 ± 0.8*	9.7 ± 1.1
	Female	11.7 ± 0.7*	12.2 ± 1.4*	11.9 ± 0.8*	8.8 ± 0.8
(b) Biochemistry parameters					
CHO (mg/dl)	Male	65 ± 6*	78 ± 6*	85 ± 9	87 ± 14
	Female	69 ± 9*	75 ± 13*	80 ± 10	73 ± 22
TG (mg/dl)	Male	99 ± 15*	103 ± 14*	105 ± 17	124 ± 25
	Female	87 ± 12*	89 ± 15*	92 ± 16	89 ± 23

CHO = cholesterol; L = lymphocytes; N = neutrophils; PLT = platelet; RBC = red blood cell; TG = triglyceride; WBC = white blood cell. * Statistical significance with control group (paired t-test, $p < 0.05$)

significantly reduced the expression of VEGF-related antiangiogenic actions in diabetic rats [36]. Administration of 6-gingerol stabilized the p-VEGFR2/VE-cadherin/β-catenin/actin complex and promoted micro-vessel normalization and suppression of tumor progression [37]. Besides, it was also found to inhibit metastasis of MDA-MB-231 human breast cancer cells [38].

The present study showed satisfactory quality, safety profile, and anti-CCA activity of the oral pharmaceutical standardized ZO extract with MTD and NOAEL of 1000 mg/kg body weight. Further investigation to elucidate molecular targets of action of ZO against CCA cell is encouraged.

Materials and Methods

Chemicals and reagents

Cisplatin and 6-gingerol (purity > 98%) were purchased from Wako Pure Chemical Industries Ltd. Ethanol and HPLC analytical

▶ **Table 2** Histopathological findings of rats after chronic administration (daily for 12 mo) of the formulated ZO powder (n = 20 per sex). Data are presented as mean ± SD values.

Microscopic Lesions		Formulated ZO powder (mg/kg body weight)					
		1000		500		250	
		Male	Female	Male	Female	Male	Female
Brain	Diffuse degeneration of the cerebral white matter	0	0	0	0	0	0
Heart	Myocardial cell necrosis	0	0	0	0	0	0
Lung	Macrophage accumulation	5 (+)	3 (+)	4 (+)	2 (+)	0	0
	Foreign body granuloma	0	0	0	0	0	0
Liver	Spotty necrosis	4 (+)	3 (+)	3 (+)	2 (+)	0	0
Kidney	Renal interstitial inflammation	4 (+)	2 (+)	0	2 (+)	0	0
Stomach	Ulcer	0	0	0	0	0	0
Spleen	Lymphoid hyperplasia	0	0	0	0	0	0
Adrenals	Cortical hyperplasia	0	0	0	0	0	0
Harderian Gland	Lymphocytic infiltration, dacryoadenitis	0	0	0	0	0	0
Urinary bladder and uterus	Cystitis and lesions	0	0	0	0	0	0
Prostate	Prostatitis	0	0	0	0	0	0
Testis	Testicular atrophy	1 (+)	0	0	0	0	0
Epididymis	No mature sperm	0	0	0	0	0	0
Vagina and cervix	Epithelial necrosis	0	1 (+)	0	1 (+)	0	0

The numbers of rats showing histopathologic changes. (+) represents minimal degree of change; (++) represents mild degree of change; (+++) represents moderate degree of change; (++++) represents severe degree of change.

grade solvents were purchased from Labscan Co., LTD. DMSO was obtained from Amresco LLC. Whatman No. 1 filter paper was purchased from GE Healthcare. The dry rhizomes of ZO were purchased from Charoensuk Pharma Supply Co., Ltd., Thailand. A voucher specimen was deposited at the herbarium of the Department of Forestry, Bangkok, Thailand (SKP 206261501).

Preparation of pharmaceutical formulation

The rhizomes of ZO were washed thoroughly with tap water, cut into small pieces, and oven-dried at 50 °C. The samples were pulverized, kept in sealed plastic bags, and stored at room temperature (25 °C). The ZO powder was macerated in 1 L of 95 % ethanol for 24 h at room temperature with occasional mechanical shaking. The extracted solvent was separated, filtered through Whatman No. 1 filter paper, evaporated under reduced pressure by rotary evaporation, and dried using spray dryer. The dried extract was weighed and stored at 4 °C until use. To prepare oral pharmaceutical formulation of the standardized ZO extract, ZO powder was mixed with an appropriate proportion of lactose, sodium lauryl sulfate, and talcum.

Quality control of the formulated standardized extract

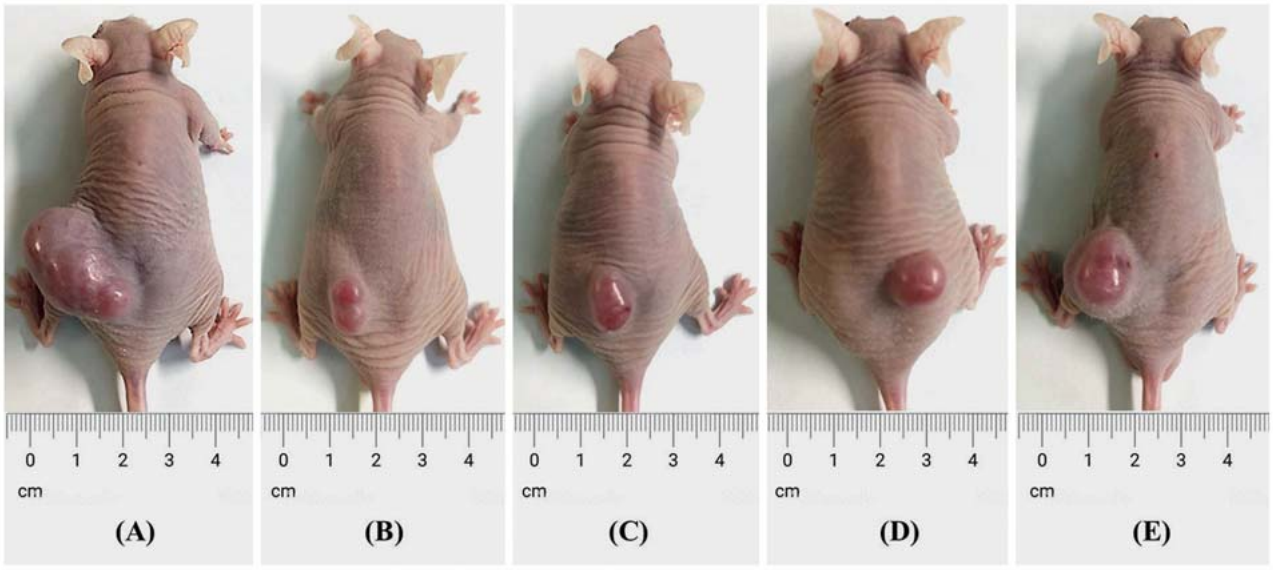
Quality control and standardization of the formulated ZO was performed using HPLC. The output was recorded as a chromatogram to identify the peak of sample constituents in formulated ZO comparing with 6-gingerol as a standard marker compound. The amount of 6-gingerol was calculated from the standard curve using the equation: $Y = 6.329X + 0.275$ (correlation coefficient = 0.999), where X represents the amount of 6-gingerol, and Y represents peak area. The chromatographic separation condition used was as follows: Thermo Hypersil Gold C18 column (4.8 × 250 mm, 5 µm); a gradient elution system consisting of a mixture of water and acetonitrile (0 min: 55/45, 8 min: 50/50, 17 min: 35/65, 32 min: 0/100, and 33–40 min: 55/45) running at the flow rate of 1 mL/min. The injection volume was 10 µL, and the absorbance wavelength was set at 282 nm.

Assessment of physicochemical properties

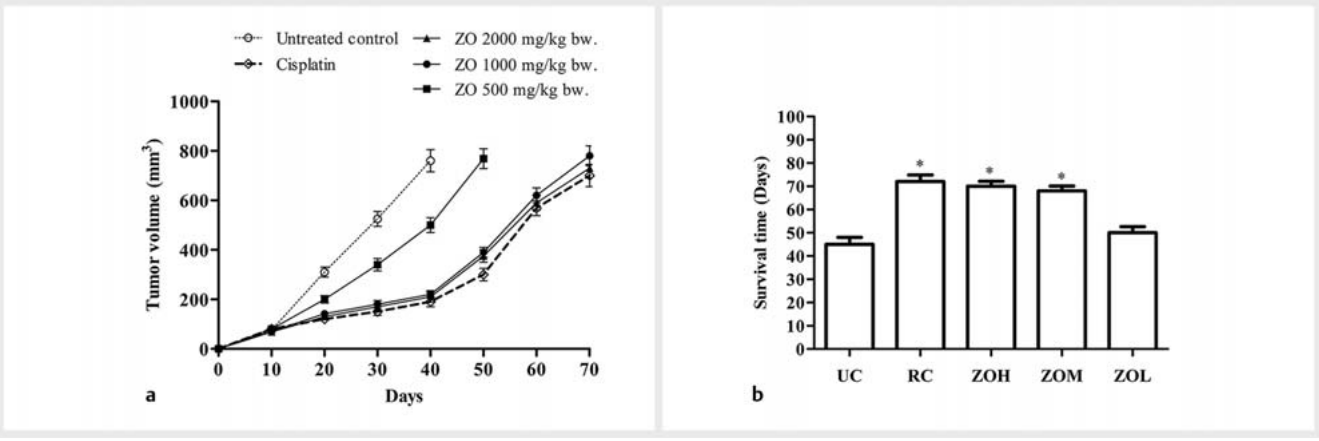
The ZO capsule formulation was evaluated for its physicochemical properties according to the Thai Herbal Pharmacopoeia 2017 criteria for water content, acid insoluble ash content, total ash content, ethanol-soluble extractive value, water-soluble extractive value, loss on drying, and volatile oil content [39].

Assessment of contaminations

Contaminations in the formulated ZO powder of heavy metals (arsenic [As], cadmium [Cd], and lead [Pb]), pesticides residues (lindane, aldrin/dieldrin, total DDT, parathion-methyl, ethion, and dichlorvos), and microorganisms (total aerobic count, total yeast and mold count, *E. coli*, and *Salmonella* spp.) were evaluated according to the Thai Herbal Pharmacopoeia 2017 [39].



▶ **Fig. 4** Anti-cholangiocacinoma activity of (**A**) untreated control and (**B**) reference control (cisplatin), in comparison with the formulated ZO powder at (**C**) high (2000 mg/kg body weight), (**D**) medium (1000 mg/kg body weight) and (**E**) low (500 mg/kg body weight) dose levels in CCA-xenografted nude mice.



▶ **Fig. 5** (**a**) Tumor volume and (**b**) survival time of untreated control (UC) and reference control (cisplatin; RC), in comparison with the formulated ZO powder at high (2000 mg/kg body weight; ZOH), medium (1000 mg/kg body weight, ZOM) and low (500 mg/kg body weight; ZOL) dose levels in cholangiocarcinoma-xenografted nude mice (N = 6 rats per group). The results are expressed as mean ± SD are significantly different compared with untreated control group (*$p < 0.05$).

Assessment of pharmaceutical properties

Flowability properties

Moisture content: The moisture content of the ZO formulated powder was measured using a moisture analyzer (Sartorius). The formulated powder (3 g) was poured into the moisture balance and distributed on the tray. The temperature of the machine was set at 130 ± 1 °C. The readings were noted when the machine automatically halted [40].

The angle of repose: The angle of repose of the ZO formulated powder was measured using a glass funnel clamped on a retort stand, which is 10 cm away from the flat surface of a bench. The formulated powder (30 g) was poured gently into the funnel and allowed to flow freely, forming a conical heap. The angle of repose was calculated from the heap of each sample using the equation: Angle of repose, tan θ = height of the circular heap/radius of the circular heap.

Bulk and tapped densities: The volume occupied by a 30-g weight of the ZO formulated powder was measured using a dry measuring cylinder. The bulk density was calculated using the formula: Bulk density = Weight of the sample/Volume of the sample.

Tapped density: The measuring cylinder was tapped 50 times on a wooden table from a height of 2 cm, and the tapped volume

▶ **Table 3** Experimental design for investigation of ant-CCA activity of the formulated ZO powder in CCA-xenografted nude mice (n = 6 per group).

Groups	Description
Group 1 (untreated control)	CCA-xenografted nude mice treated with normal saline (NSS)
Group 2 (reference control)	CCA-xenografted nude mice treated with reference drug, cisplatin
Group 3 (low dose)	CCA-xenografted nude mice treated with 500 mg/kg body weight of the formulated ZO powder
Group 4 (medium dose level)	CCA-xenografted nude mice treated with 1000 mg/kg body weight of the formulated ZO powder
Group 5 (high dose level)	CCA-xenografted nude mice treated with 2000 mg/kg body weight of ZO formulation

was noted. The tapped density was calculated as: Tapped density = Weight of sample/Tapped volume of sample.

Determination of Carr's index: Carr's index was determined using results obtained for both bulk density and tapped densities: Carr's index (%) = [(Tapped density – Bulk density)/Tapped density] × 100.

Determination of Hausner's ratio: Hausner's ratio was determined using the result obtained for both bulk densities and tapped densities according to the formula: Hausner's ratio = Tapped density/Bulk density.

Weight variation

The average weight of each formulated capsule was determined by 20 randomly selected filled capsules and weighted collectively, and the average weight of each formulated capsule (mean ± SD), including weight variation (% RSD: relative standard deviation) was determined.

Disintegration test

The disintegration of the formulated capsules was evaluated according to the European Pharmacopoeia with modifications [41]. Six capsules were put into the disintegration tester along with sliding discs. The temperature of the water was maintained at 37 ± 2 °C. The disintegrating time required for each of the 6 capsules was recorded. All of the 6 capsules must be disintegrated within 30 min [42].

Dissolution test

The dissolution of the formulated ZO capsules was evaluated according to the US Pharmacopoeia [40] using apparatus II tester. The test condition applied was as follows: phosphate buffer pH 6.8 (900 mL, 37 ± 0.5 °C) as dissolution medium, and 50 *rpm* paddle speed. Aliquots (5 mL each) of dissolution medium were collected at regular time intervals (10, 15, 20, 30, 45, 60, 120, and 180 min) and filtered through Whatman filter paper (No. 1). The amount of 6-gingerol was determined using HPLC as described above [42].

Stability test

Stability test of the formulated capsules at different periods (0, 1, 2, 3, and 6 mo) was examined at accelerated condition (40 °C ± 2 °C and 75 ± 5 % relative humidity [RH]) in the stability chamber. The stability at normal condition (30 ± 2° and 75 ± 5 % RH) was examined at 0, 1, 2, 3, 6, and 12 mo [43]. The weight of the formulated ZO capsule was recorded, and the amount of 6-gingerol in the formulated ZO was measured using HPLC as previously described.

Assessment of toxicity and anti-CCA activity

Animal

BALB/cAJcl-nu/nu nude mice (6 wk of age, weighing 20–25 g) and Sprague Dawley rats (6 wk of age, weighing 150–200 g) were used in the experiments. All animals were supplied by Nomura Siam International Co., Ltd., Thailand. The experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health publication number 85–123). Approval of the study protocol was obtained from the Ethics Committee for Research in Animals, Thammasat University (Approval number 008/2560, 7 August 2017). All animals were housed under standard conditions and fed with a stock diet and water *ad libitum*.

Toxicity tests

Acute, subacute, and chronic toxicity tests were performed to obtain the 3-dose levels (maximum tolerated, medium, and low dose levels) of the formulated ZO powder that did not cause unacceptable signs of toxicity or death [44–46]. The rats (5 males and 5 females each for acute and subacute toxicity, and 20 males and 20 females of chronic toxicity) were orally administered (*via* gastric gavage) 3-dose levels of the formulated ZO powder (resuspended in distilled water). The maximum dose started from 5000 mg/kg body weight. The control animals were fed with distilled water. Animals were strictly observed for signs of toxicity during the first 30 min, periodically during the first 24 h, and then daily for 14 days (acute toxicity), 28 days (subacute toxicity), or 12 mo (chronic toxicity). At the end of the observational period (on days 15, 29, and 366), all animals were sacrificed by euthanasia with isoflurane anesthesia. Vital organs (brain, heart, kidneys, liver, spleen, stomach, large and small intestine, lungs, adrenals, harderian gland, urinary bladder and uterus, prostate, testis, epididymis, and vagina, and cervix) were removed from all animals for gross and histopathological examination. Blood samples were collected in vacutainer tubes (with and without EDTA anticoagulant) at the terminal stage following euthanasia of rats for hematology tests (red cell series, white cell series, and platelet counts) and serum chemistry tests (aspartate transaminase [AST], alanine transaminase [ALT], alkaline phosphatase [AL], creatine kinase [CK], lactate dehydrogenase [LDH], total proteins, albumin, globulin, glucose, triglycerides, total cholesterol, urea, creatinine, and uric acid). The hematology and serum chemistry parameters were determined using ProCyte Dx hematology analyzer (IDEXX) and automated biochemical analyzer, Cobas c311 (Roche Diagnostics), respectively.

Anti-CCA activity

The anti-CCA activity of the formulated ZO powder was evaluated using the CCA-xenografted mouse model. The CCA cell line CL-6 was used for xenografting in all nude mice. Cells were removed from culture flask by cell scraper and collected in a 50 mL conical tube. Following centrifugation (700 × *g* for 5 min), the supernatant was removed, and cells were resuspended in 5 mL of complete media, and the number of cells was counted using hemocytochamber. Cells for injection (subcutaneous) were prepared by diluting cell suspension to obtain 1 000 000 cells/200 µl normal saline (0.85 % NaCl). Tumors were allowed to develop until they reach approximately 50 mm^3 tumor volume. The tumor volume was measured using a digital caliper, and the body weight was recorded once every 2 days. The animals were divided into 5 groups (6 males for each group, matched-pair according to tumor size, and body weight) (▶ **Table 3**).

The formulated ZO powder was fed to animals (group 3–5) by oral feeding using gastric gavage (0.2 mL) daily for 30 days. Animals in the untreated (group 1) and cisplatin-treated (group 2) control groups were fed with an equal volume of normal saline daily for 30 doses and cisplatin (50 µg/mL) daily for 14 doses, respectively. Tumor size was measured in 2 linear dimensions (maximum longitudinal and transverse diameters) using digital calipers with an accuracy of 0.01 mm. The tumor volume was calculated using the formula: Tumor volume = (length) × $(width)^2/2$.

The primary endpoint parameters for anti-CCA activity included tumor growth inhibition, inhibitory activity on lung metastasis, and survival time. The secondary endpoint parameters were body weight and food and water consumption. At sacrifice, the tumors were recovered, and the wet weight of each tumor was recorded. Separate portions of each tumor were fixed in formalin for hematoxylin and eosin staining.

Statistical analysis

Statistical analysis was performed using SPSS statistical package 18.0 (SPSS). All quantitative variables were presented as mean ± SD values. Comparison of all the quantitative variables between the groups given test materials and reference drugs were performed using unpaired (for 2 independent variables) or paired (for 2 dependent variables) t-test at a significance level of $\alpha = 0.05$.

Supporting information

Results of all formulation parameters and hematological and biochemical parameters of chronic toxicity study of the formulated ZO powder are available as Supporting Information.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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